VESTAR, INC.

October 21, 1992

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REF: Triannual Report August, 1992 Contract #N00014-90-C-0195

Contract work for the May - August time period was directed at evaluating the Vestar 7:10:3:0.4 formulation and optimizing the pilot scale processing parameters to replicate early sonication results. This included evaluation of the hydration process and preliminary toxicity studies. Work on developing an accurate and rugged test for determination of %met-hemoglobin was continued. A summary of relevant experiments is presented below:

1. Homogenization Pressure May 1992

Homogenization of 7:10:3:0.4 lipid with a buffer hydration using 10 mM phosphate, 9% sucrose buffer was performed at 3000 and 4000 psi and 20-25° C to evaluate the effect of increased temperature and pressure on size of liposomes. The increased pressure and temperature slightly increased amount of %met-hemoglobin but improved the size distribution and permitted filtration through 1.2 μm and 0.8 μm filters.

2. Murine Toxicity Study May 1992

Doses of the above LEH with 0.71 and 0.57 g/Kg of rHb 1.1 were administered to Balb/C female mice to determine LD_{50}. Empty liposomes were administered as a control. Weight loss and deaths were measured. The empty liposomes caused no

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measurable toxicity and the LEH LD_{50} was between 0.57 and 0.71 g/Kg.

3. NaCl Aggregation Study May 1992

NaCl was added to Vestar 7:10:3:0.4 LEH in phosphate/sucrose buffer to assess the effect of buffer ionic strength on LEH aggregation as determined by size and precipitation. The mean size increased with increasing NaCl concentration and precipitate was evident at concentrations of 100 and 200 mM.

4. P_{50} of Healthy Rodent Blood May 1992

P_{50} of Balb/C female mouse and Sprague-Dawley rat was measured to help understand why mice in the above toxicity study turned blue. P_{50}s were 40 and 38, respectively.

5. Toxicity Study of Navy Formulation LEH May 1992

A typical pilot scale lot of Navy Formulation LEH with bovine Hb was produced to supply material for a toxicity study to determine LD_{50} of Balb/C female mice. Doses of LEH at 0.65, 0.75, and 0.85 g/Kg of bovine hemoglobin were administered. LD_{50}s of both LEH and free bovine Hb were found to be greater than 0.85 g/Kg.

6. Substitute HSPC for DSPC May 1992

LEH with rHb 1.1 and the Vestar 7:10:3:0.4 lipid formulation was produced to evaluate the effect of the less expensive HSPC on physical properties and toxicity. The HSPC LEH was similar to DSPC material and showed promise by filtering easily through 5 μm and 0.8 μm filters and with more difficulty through 0.65 and 0.45 μm filters. LD_{50} of Balb/C female mice was 0.57 g/Kg.

7. Small Scale Homogenizer I June & August 1992

Because of concern over the supply of hemoglobin, two small homogenizer units (40 mL and 140 mL process volumes) were evaluated. Both units were effective in producing LEH typical of that produced in larger units.

8. Hydrated Lipid Viscosity Study June 1992

Observed differences in effect of mixing during buffer hydration on the NRL 10:9:1:0.4 and Vestar 7:10:3:0.4 formulations and in size of final LEH led to an investigation of viscosity behavior of the lipids. The viscosity of the 7:10:3 lipid mixture decreased with mixing while the 10:9:1 formulation was not affected. This was consistent with size behavior; while the 10:9:1 is insensitive to hydration method, 7:10:3 lipid hydrated with buffer and mixed constantly produces an improved size distribution.

9. Development of Met-Hb Assay May-Aug 1992

Attempts to develop a simple methemoglobin assay by applying multi-component analysis to absorbance spectra of hemoglobin was unsuccessful primarily because hemoglobin standards were not available.

10. Hb Stability Study May-Aug 1992

Stability studies comparing bovine Hb versus rHb 1.1 in free and liposomal form indicate that the rHb 1.1 is converted to methemoglobin much more quickly than the bovine hemoglobin. Addition of glycerol (>25%w/v) stabilizes both hemoglobins significantly and may be useful in assay development and processing.

11. Hb Position in Lipid Bilayer May-Aug 1992

Initial studies to determine the position of Hb molecules with respect to the liposomal bilayer have focused on the use of fluorescent probes which will label the Hb. Currently, the optimum conditions for labeling Hb are being defined.

12. Effect of Glycerol on Liposomes May-Aug 1992

Liposomes (2:1 HSPC:Cholesterol) were prepared in phosphate buffered glycerol solutions at different concentrations of glycerol (5%-60%). The size distributions were typically less than 100 nm even with 60% glycerol. This may prove to be useful in processing liposomal Hb with glycerol as a stabilizing agent.

13. Capillary Electrophoresis Analysis of Hb May-Aug 1992

Preliminary analysis of Hb by capillary electrophoresis yielded encouraging results. Two major peaks were resolved which tentatively were assigned to the oxy and met forms of Hb. Several minor peaks were also observed. These are thought to be further degradation products (i.e. hemichromes).

14. Comparative Toxicity Study August 1992

Four batches of LEH were produced with all combinations of 10:9:1:0.4 and 7:10:3:0.4 lipid mixtures and rHb 1.1 and bovine Hb. Lipids were hydrated directly in the Hb in phosphate/saline buffer. One batch of Vestar 7:10:3:0.4 with a buffer hydration and phosphate/sucrose buffer was made. The study is intended to pin down the cause of greater toxicity that has been seen in the Vestar 7:10:3:0.4 lipid/rHb 1.1 formulation. Testing has not yet been completed.

Investigation of formulations and processing conditions will be continued as will product characterization studies. We are awaiting the results from the comparative toxicity study and discussions with NRL personnel at the meeting in November to define the emphasis of future research.

Sincerely,

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